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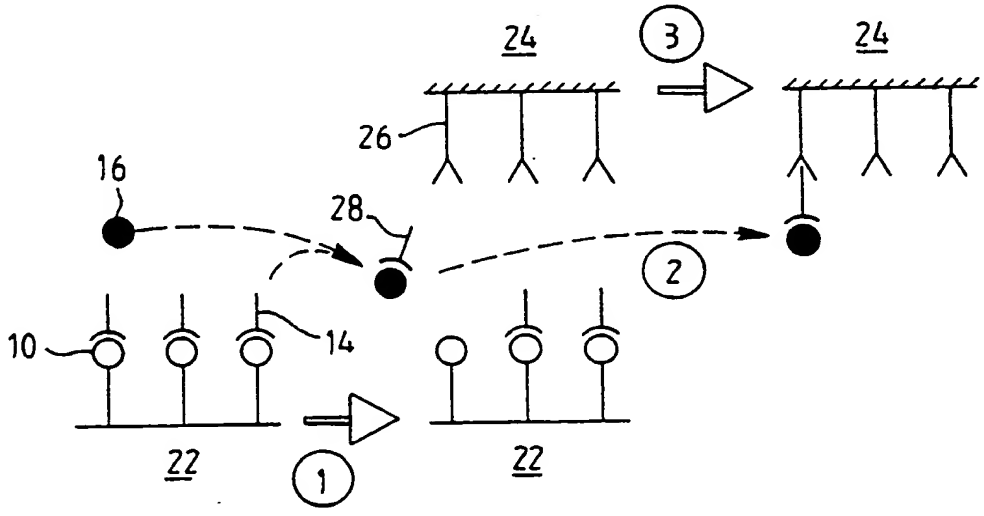

(57) Abstract

A method of assaying for an analyte by means of surface plasmon resonance spectroscopy involves the use of separate displacement and detection surfaces. In one example, the displacement surface (22) has reversibly bound to it a specific binding partner (14) of the analyte; and the detection surface (24) carries an antibody (26) to the specific binding partner. When a fluid sample is flowed successively over the displacement and the detection surfaces, the analyte (16) in the sample causes the specific binding partner to be displaced and to become bound on the detection surface. Systems employing one or more enzymes can be used to amplify the SPRS signal.

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ASSAY METHOD

This invention concerns methods of assaying for analytes using the technique of surface plasmon resonance spectrometry (SPRS). The method is applicable to analytes generally, but is likely to be of particular interest where the analyte is a hapten (a small molecule capable of being bound by antibody but not of itself immunogenic).

The phenomenon on SPR is well known and will not be described in detail. (See EPA 305109 for example.) Briefly, the intensity of monochromatic plane-polarised light (conveniently obtained from a laser) reflected from the interface between an optically transparent material, e.g. glass, and metal depends on the refractive index of material in a thin layer, at most a few hundred nm thick, on the downstream side of the metal. Accordingly, by measuring changes in intensity of reflected light an indication can be obtained of changes in refractive index of material on the metal. The intensity of reflected light also varies with the angle of incidence, and reflectivity drops sharply to a minimum at a particular angle characteristic of the equipment. The metal surface is generally of silver, although this is not critical to the invention.

The immunoassay of haptens by Surface Plasmon Resonance Spectrometry (SPRS) poses a particular problem because the haptens are, by definition of low molecular weight and therefore cause only very small changes in refractive index when they bind to or dissociate from an antibody-coated SPRS silver-coated surface. This problem is addressed in International

Patent specification WO 89/08260.

Two ways around this problem are illustrated in Figure 1 of the attached drawings. In 1 (a), the hapten 10 is immobilised to the silver surface 12 used for SPRS detection, and binds the corresponding antibody 14. Introduction of free hapten 16 (whose concentration it is wished to determine) displaces antibody by competing with surface bound hapten. This displacement of antibody from the surface is detected as an SPRS signal. In 1 (b), the antibody 14 is bound to the surface 12 and binds a conjugate 18 of the hapten 10 and (typically) a protein 20 of sufficient molecular weight to yield a significant SPRS signal on displacement of the conjugate by added free hapten.

However, this arrangement is not always very sensitive. This invention provides a way of increasing the sensitivity.

This invention provides a method an assaying for an analyte, by the use of a displacement surface carrying immobilised thereon a first reagent to which a second reagent is reversibly bound, and of a detection surface carrying immobilised thereon a third reagent,

wherein either (a) the first reagent is an analogue of the analyte and the second reagent is a specific binding partner of the analyte, or (b) the first reagent is a specific binding partner of the analyte and the second reagent is an analogue of the analyte,

which method comprises contacting a fluid sample containing the analyte successively with the displacement surface and the detection surface, whereby the second reagent is released from the displacement surface and causes, directly or indirectly, a reaction which alters the third reagent in a manner related to the concentration of the analyte in the sample,

wherein there is used as the

detection surface a metallic layer applied to a block of material transparent to electromagnetic radiation, alteration of the third reagent on the detection surface being assayed by surface plasmon resonance spectroscopy.

An analogue of the analyte is a substance which competes with the analyte for binding to a specific binder thereof. Often the analogue structure will be arranged to be as near as possible or even completely identical to the analyte. The use in assays of analyte analogues is well known.

Two embodiments of the invention have been designated a) and b). In a), the first reagent immobilised on the displacement surface is an analogue of the analyte, preferably the analyte itself carried on a suitable spacer molecule (generally a macromolecule such as a protein). The second reagent, reversibly bound to the first reagent, is a specific binding partner of the analyte, most usually an antibody to the analyte, or a conjugate of such specific binding partner with an enzyme.

In b), the first reagent, immobilised on the displacement surface, is a specific binder for the analyte, generally an antibody to the analyte. The second reagent, reversibly bound to the first reagent, is then an analogue of the analyte, preferably a conjugate of the analyte or an analogue thereof with a macromolecule such as a protein or an enzyme.

The advantages of separating the first surface, where analyte displaces the second reagent, from the second surface, where alteration of the third reagent is detected by SPR, are manifold including:

- the first surface can be optimised for its intended purpose. It needs to be neither silver nor planar. Indeed it can be beads suspended in the surrounding medium to give a greatly increased area for

the displacement reaction. Or it can be provided by a layer of fibres overlying the detection surface, which fibres may be in the form of a woven or non-woven fabric or cloth. Or it can be provided by a body of particles.

- the detection surface can also be optimised for its purpose. Because it is spatially separate from the displacement surface, the properties of the third reagent immobilised thereon can be chosen to maximise the change in SPR signal caused by its alteration. The SPR signal is not masked by the presence of other immobilised reagents required for the assay.

- the physical separation of the displacement and detection surfaces in space allows the possibility of a temporal separation also, with possible analytical and experimental advantages. This includes processing the displacement to equilibrium, followed by detection to enhance sensitivity.

The nature of the displacement surface is not critical to the invention, it being merely necessary that the first reagent be immobilised thereon for the duration of the assay. However, as noted above, it is preferred that the displacement surface be provided by a layer of fibres overlying the detection surface. Fibres may be woven or non-woven into a thin layer of fabric or cloth or paper closely opposed to the detection surface. The advantage of using a thin layer of cloth, fabric or paper in this way are several:

- ease of manufacture
- wide choice of material and of chemistry for immobilisation of assay reagents
- wide choice of materials to provide large surface area, high porosity, wettability and access of solid molecules to bound reagents
- only a few points of physical contact

between the cloth or fabric layer and the refractive index sensitive zone (about 100 nm thick) extending from the detection surface into the bulk liquid phase

- ability to weave or make up a cloth or fabric from more than one thread or fibre, where each type of thread has immobilised upon it molecules that are required to be kept separate from those on other types

- ability to retain drops of analyte solution in contact with itself and the adjacent detection surface.

Alternatively, a body of particles or small beads, typically a few microns in diameter, can replace the fabric or cloth and be held adjacent the detection surface. The particles can be arranged as a layer or a bed or as a column. A column may contain an inert particulate substrate, such as for example resin beads, porous ceramic beads or gels, to which are bound the first reagent. Alternatively again, such particles or beads can be immobilised in the interstices of a cloth or fabric, with the fibres of the cloth or fabric carrying one or more assay reagents and the particles or beads carrying one or more other assay reagents.

The detection surface is of a kind typically used for SPRS. There needs to be a pathway for the second reagent (or other assay reagents derived from it) to translocate (e.g. by diffusion, flow, electrophoresis or carriage) from the displacement surface to the detection surface. The detection surface carries immobilised thereon a third reagent which is altered, as described in more detail below, in a manner related to the concentration of the analyte in the sample.

In one embodiment, the third reagent is a specific binder designed to capture second reagent translocated from the displacement surface. Various

capture mechanisms are available, depending on the nature of the second reagent:

- an antibody against the second reagent;
- protein A (or Protein G) when the second reagent is itself an antibody;
- streptavidin or avidin when the second reagent has been labelled with biotin.
- a specific binder for the analyte, particularly in the case where the analyte is a macromolecule such as an antibody.

Translocation of the second reagent occurs in a manner, e.g. to an extent or at a rate, which is related to the concentration of the analyte in the sample. It may be monitored by SPRS, e.g. as the rate of change of reflectivity or as the total change in reflectivity over a given period.

In a preferred embodiment of the invention, the second reagent is a conjugate of an enzyme, which is used to catalyse a secondary reaction. Because one molecule of enzyme is capable of successively catalysing reaction of many substrate molecules, this arrangement can be used to amplify the signal generated by the analyte, and thus to improve the speed or sensitivity of the assay. Use of two or more enzyme systems can provide further greatly enhanced signal amplification. When the second reagent comprises an enzyme, there are two possibilities for the third reagent on the detection surface:-

a) A third reagent can be a specific binder designed to capture some species which has been released, either directly or indirectly, by the enzyme into solution. To maximise the SPR signal, this species may be a material of high (or alternatively low) refractive index relative to the bulk material adjacent the detection surface. Various capture mechanisms are available, depending on the nature of

the species, as listed above for the case where the second reagent is directly captured by the third reagent.

b) The third reagent is a species whose release into solution from the detection surface is catalysed by an enzyme. This enzyme may be either the enzyme of the second reagent, or some other enzyme as described below. To enhance the SPR signal, the third reagent is generally a material of high (or alternatively low) refractive index relative to the bulk material adjacent the detection surface. Or the third reagent may itself comprise an enzyme forming part of a multiple enzyme amplification system.

Reference is directed to the accompanying drawings, in which each of Figures 2, 3, 4, 5 and 6 is a diagrammatic representation of a different system according to the invention.

In Figure 2, the second reagent is simply translocated from a displacement surface to a detection surface.

In each of Figures 3 and 4, the second reagent comprises an enzyme which causes a high refractive index species to be removed from (Figure 3) or attached to (Figure 4) a detection surface.

Figure 5 illustrates a cascade amplification system involving two enzymes and Figure 6 illustrates a crossed feedback amplification also involving two enzymes.

Figures 7 and 8 are diagrams of the equipment used for Examples 1 and 2.

Figure 2 illustrates two systems according to the invention. These are drawn to correspond to those shown in Figure 1, and the reference numerals are, so far as possible, the same.

Referring to Figure 2 (a), a displacement surface 22 carries immobilised thereon an analyte

analogue 10, to which antibodies 14 are reversibly bound. A detection surface 24 carries immobilised thereon antibodies 26 to the (Fc portion of the) antibodies 14. In step 1, a fluid sample containing an analyte 16 is brought into contact with the displacement surface 22. The analyte 16 captures some of the antibody molecules 14 forming conjugates 28. These conjugates are caused to translocate to the detection surface 24 where they become bound to the antibodies 26. The change in refractive index, resulting from binding of these conjugate molecules to the detection surface, is monitored by SPRS.

In Figure 2 (b), a displacement surface 22 carries immobilised thereon an antibody 14 to the analyte. Reversibly bound to the antibody is a conjugate 18 of an analyte analogue 10 with a protein 20. A detection surface 24 carries immobilised thereon an antibody 30 to the protein 20. In step 1, a fluid sample containing the analyte is contacted with the displacement surface 22 and the analyte displaces a proportion of the conjugate 18. In steps 2 and 3, this conjugate translocates to the detection surface 24, where it is captured by the antibody 30. The change in refractive index, caused by capture of the conjugate 18 at the detection surface, is monitored by SPRS.

Figures 3 to 6 are more simply drawn, with the changes taking place during the course of the assay merely shown by arrows. In each case, the first reagent attached to the displacement surface is an analyte analogue, as in Figure 2 a). In each case, it is easy to envisage a related system in which the first reagent attached to the displacement surface is an antibody to the analyte, as shown in Figure 2 b).

Referring to Figure 3, a displacement surface 22 carries immobilised thereon an analyte analogue 32

as the first reagent. Reversibly bound to this is a conjugate 34 of an antibody to the analyte with an enzyme E. The detection surface 24 carries a high refractive index species (R1) 36 immobilised thereon by means of a linker (S) 38 which is a substrate for the enzyme, and which is cleavable.

When a fluid sample containing an analyte 42 is brought into contact with the displacement surface 22, the analyte causes release of some of the antibody/enzyme conjugate 34 into solution. This conjugate translocates to the detection surface 24, where the enzyme cleaves the substrate 38 and releases the high refractive index species 36 into solution. Amplification results from the fact that each molecule of enzyme releases several molecules of high refractive index species into solution.

Referring to Figure 4, a displacement surface comprises two regions, designated 22 and 22´. The first region 22 carries analyte analogue 32 immobilised thereon, to which an analyte antibody/enzyme conjugate 34 is reversibly attached. The second displacement region 22´ carries a high refractive index species (R1) 36 immobilised thereon by means of a substrate (S) 38 for the enzyme which is cleavable by the enzyme. A detection surface 24 carries a specific binder 40 for the high refractive index species 36.

Addition of a fluid sample containing an analyte 42 causes release of some of the antibody enzyme conjugate from the displacement surface 22 into solution. This migrates to region 22´ of the displacement surface, where the enzyme catalyses the release into solution of the high refractive index species. This in turn translocates to the detection surface 24 where it is captured by its specific binder. Signal amplification results from the fact that each molecule of enzyme can catalyse release into solution

of several molecules of the high refractive index species.

There can of course be difficulties in immobilising both an enzyme (as its antibody conjugate) and its substrate in close proximity to each other on the same surface, because the enzyme will attack its substrate during the immobilisation process. However, by immobilising the antibody/enzyme conjugate on one thread and the substrate on another, a cloth containing both can then be woven. Alternatively, the two reagents can be immobilised on separate bead or fibre populations which are then mixed and layered onto the detection surface.

The amplification achieved by a single enzyme can be greatly increased by cascading two or more enzymes in stages, such that the output of one stage provides a further stage of amplification. An example of such a system in shown in Figure 5, where as previously the displacement surface comprises two regions designated 22 and 22'. Immobilised on the region 22 is an analyte analogue 44, to which an analyte antibody/first enzyme (E_1) conjugate 46 is reversibly attached. On the region 22', a second enzyme (E_2) 48 is immobilised by means of a substrate (S_1) 50 for the first enzyme which is cleavable by the first enzyme. On the detection surface 24, a high refractive index species (R_1) 52 is immobilised by means of a substrate (S_2) 54 for the second enzyme which is cleavable by the second enzyme.

A fluid sample containing the analyte 56 is brought into contact with the displacement surface. The analyte causes displacement into solution of some of the antibody/first enzyme conjugate. The first enzyme cleaves its substrate, thus releasing some of the second enzyme into solution. The second enzyme translocates to the detection surface 24 where it

cleaves its substrate, releasing the high refractive index species into solution. The system thus involves two enzyme amplification steps in series. Other cascade amplification systems, e.g. along the lines of the system shown in Figure 4, or involving three or more enzymes, can readily be envisaged.

Figure 6 shows a crossed feedback amplification system involving two enzymes, in which the displacement surface comprises three regions designated 22, 22´ and 22´´. The region 22 carries immobilised analyte analogue 44, to which an analyte antibody/first enzyme conjugate 46 is attached. The region 22´ carries a second enzyme 48 immobilised by means of a substrate 50 for the first enzyme which is cleavable by the first enzyme. The region 22´´ carries the first enzyme 58 immobilised by means of a substrate 54 for the second enzyme and which is cleavable by the second enzyme. The detection surface 24 carries a high refractive index species 52 immobilised by means of a substrate for the second enzyme 54 and which is cleavable by the second enzyme.

When a sample containing the analyte 56 is brought into contact with the displacement surface, the analyte causes release of some of the antibody/first enzyme conjugate into solution. When it reaches the region 22´, the first enzyme catalyses the release into solution of the second enzyme. At least regions 22´ and 22´´ are in communication by bulk phase solvent, such that the released second enzyme can diffuse (or be translocated by some other means) to the region 22´´ and can there release more first enzyme into solution. Conversely, released first enzyme can move from region 22´´ to region 22´.

Thus, introduction of first enzyme in solution into the system initiates an autocatalytic reaction in which each enzyme catalyses production of

the other. In addition, the second enzyme translocates to the detection surface 24 where it catalyses release into solution of the high refractive index species. The positive feedback gain is very high, such that a single molecule of first enzyme in solution may be sufficient to generate an easily observed SPR signal.

Many variations of this system are possible. It is not necessary that the first enzymes attached to regions 22 and 22'' of the displacement surface be identical, provided that both are capable of cleaving the first substrate. It is not necessary that the second substrate attached to region 22' of the displacement surface and to the detection surface 24 be identical, provided that both are cleavable by the second enzyme. The high refractive index species can be attached to the detection surface 24 by the first substrate rather than the second substrate. If instead of the high refractive index species, the first enzyme is immobilised on the detection surface 24 by means of the second substrate, then the region 22'' of the displacement surface can be dispensed with altogether.

The use of a woven cloth for implementing a crossed feedback amplification system provides an effective way of keeping the first enzyme away from its substrate and the second enzyme away from its substrate during their immobilisation on individual threads, yet enables their close approximation when the three threads are woven together into a cloth.

Alternatively, the three different regions can be provided by three separate populations of beads or fibres which are then brought together as a layer on the detection surface. Or the beads or fibres can be trapped in a porous fabric or membrane which is applied to the detection surface.

The following Examples illustrate the

invention.

Example 1

This example uses an immunoaffinity column linked in series with a standard SPR flow cell.

Construction

Mouse anti-thyroxine monoclonal antibody (α T4 MAb) was covalently bound to a chromatography column support material. This matrix was then packed into a short length of tubing (0.5 ml) to form a column. The column was then incorporated into the flow path of an SPR rig set-up.

The fluid addition part of the SPR rig set-up is shown in Figure 7. This comprises a syringe pump 70, a column 71, a peristaltic pump 72, a first tap 73, a second tap 74, a flow cell 75, a silver slide 76, the parts being connected by flow paths a, b, c, d, e, f, g and h, with flow paths g and h leading to waste 77.

Operation of fluid flow

Fluid flow patterns are described with reference to Figure 7. Each flow path serves a specific purpose, and the column paths used are listed below. Within the body of the text describing the assay format the flow paths will be referred to by these headings (i-v).

i. **Normal Use.**

Fluid is pumped using the syringe pump 70 only, with the peristaltic pump 72 disengaged. Fluid flows via paths: a.->b.->e.->f.->h.

ii. **Addition of reagents for recycling.**

With the peristaltic pump disengaged fluid is flowed through paths: a.->b.->e.->g. This flow direction avoids the silver surface 76 and is also used for some wash cycles.

iii. Recycling reagents via column.

The peristaltic pump is engaged and used to flow fluids along paths: b.->c.->d.->e.

iv. Elution of assay reagents.

Fluid is pumped using the syringe pump only, with the peristaltic pump disengaged. Fluid flows via paths: a.->b.->e.->f.->h. Reagents which have been recycled through the column 71 can be flowed across the silver slide using this flow path.

v. Washing column.

Flow path: a.->c.->d.->g., is used to remove excess reagents from column.

Methods

Reverse triiodothyronine covalently coupled to human IgG antibody (rT3-hIgG) is bound by the mouse anti-T4 on the column matrix. The addition of T4 results in the displacement of the rT3-hIgG conjugate since the anti-T4 antibody has a higher affinity for the T4 than for the rT3-hIgG.

The method used to measure the displacement of rT3-IgG conjugate by added T4, from a column containing monoclonal anti-T4 is described below.

i. Coating silver surface.

Anti-human IgG polyclonal antibody (α hIgG) is pumped across the silver surface (Flow path i). This is followed by a wash cycle.

ii. Column pre-treatment sequence.

Reverse T3 covalently bound to human IgG antibody (rT3-hIgG) was added through the fluid path (Flow path ii) and then re-cycled (Flow path iii) such that at least three complete cycles were achieved. The rT3-hIgG will be bound by the α T4 MAb on the column. At the end of the re-cycling period washes were used to remove all surplus reagents from both the column (Flow path v) and the peristaltic pump tubing (Flow path ii).

iii. Displacement Reaction.

T4 solution was added through the fluid path (Flow path ii) and then re-cycled (Flow path iii) such that at least three complete cycles were achieved. At the end of the re-cycling period the solution which had been re-cycled was pumped across the silver surface (Flow path iv). The α T4 MAb has a higher affinity for the T4 antigen than for the rT3-hIgG and so the rT3-hIgG will have been displaced. The displaced rT3-hIgG is then bound by the α hIgG on the silver surface and this binding will be detected via SPR. This assay gives a measure of the amount of T4 present in a sample, where the signal produced is directly proportional to the T4 concentration in the sample.

Results

Two sets of column material were prepared and used in separate experiments and the data obtained are summarised below. SPR signal units are expressed as milli-degrees of angular shift (mDA).

T4 Added (μM)	Relative Rate of Antigen Binding (mDA sec^{-1})	
	Occasion 1	Occasion 2
10	5.433	8.948
5	4.016	4.749
2.5	2.418	3.133
0	1	1

Example 2

This method uses two channels of flow cell, one containing the displacement surface and the other the detection surface, the two channels being run either in parallel or connected in series.

Construction

The flow paths for the two channels of the flow cell were set up as shown in Figure 8.

This comprises a first channel 80 comprising a syringe pump 81 and a displacement surface 82; and a second channel 83 comprising a syringe pump 84 and a detection surface 85, the parts being connected by flow paths a, b, c, d, e, f and g, with flow paths c and g leading to waste 86.

Operation of fluid flow

Fluid flow patterns are described with reference to Figure 8. Within the body of the text describing the assay format the flow paths will be referred to by these headings (i-iii).

i. **Preparation of displacement surface.**

Fluid is flowed via paths: a.->b.->c.

ii. **Preparation of detection surface.**

Fluid is flowed via paths: e.->f.->g.

iii. **Displacement and detection of displaced species.**

Fluid is flowed via paths: a.->b.->d.->f.->g.

These flow paths (i-iii) are also used for wash cycles.

Methods

rT3-hIgG is immobilised in one of the flow cell channels and α T4 MAb is then bound to it. On addition of T4 to this complex displacement occurs as described in Example 1.

The method used to measure the displacement

of the α T4 MAb is described below.

i. Preparation of displacement surface.

The displacement surface in channel 1 is coated by pumping rT3-hIgG through the channel (Flow path i). This is followed by wash and block cycles using the same flow path. The α T4 MAb is then pumped through channel 1 where it binds to the immobilised rT3-hIgG. (Flow path i).

ii. Preparation of detection surface.

The silver surface in channel 2 is coated by pumping anti-mouse IgG polyclonal antibody (α mIgG) through the channel (Flow path ii). This is followed by wash and block cycles using the same flow path.

iii. Displacement and detection of anti-T4 antibody.

T4 solution was added to the two channels connected in series such that it flowed across the displacement surface followed by the detection surface (Flow path iii). The α T4 MAb has a higher affinity for the T4 than for the rT3-hIgG and hence is displaced from the immobilised rT3-hIgG. The displaced α T4 MAb then flows into channel 2 where it is captured by the immobilised α mIgG. This capture reaction is detected by SPR. The assay gives a measure of the amount of T4 present in the sample, the signal produced being directly proportional to the T4 concentration.

Results

Reagents were prepared on two occasions and used in separate experiments and the data generated are summarised below. SPR signal units are expressed as milli-degrees of angular shift (mDA).

T4 Added (µM)	Change in mDA due to aT4 MAb binding	
	Occasion 1	Occasion 2
0	1.39	5.76
10	26.3	19.3
50	69.8	54.4

CLAIMS

1. A method an assaying for an analyte, by the use of a displacement surface carrying immobilised thereon a first reagent to which a second reagent is reversibly bound, and of a detection surface carrying immobilised thereon a third reagent,

wherein either (a) the first reagent is an analogue of the analyte and the second reagent is a specific binding partner of the analyte, or (b) the first reagent is a specific binding partner of the analyte and the second reagent is an analogue of the analyte,

which method comprises contacting a fluid sample containing the analyte successively with the displacement surface and the detection surface, whereby the second reagent is released from the displacement surface and causes, directly or indirectly, a reaction which alters the third reagent in a manner related to the concentration of the analyte in the sample,

wherein there is used as the detection surface a metallic layer applied to a block of material transparent to electromagnetic radiation, alteration of the third reagent on the detection surface being assayed by surface plasmon resonance spectroscopy.

2. A method as claimed in claim 1, wherein the third reagent is a specific binder for the second reagent which captures the second reagent which is released from the displacement surface.

3. A method as claimed in claim 1, wherein the second reagent is a conjugate of an enzyme with (a) a specific binder partner of the analyte, or (b) an analogue of the analyte.

4. A method as claimed in claim 3, wherein the third reagent comprises a substrate for an enzyme.

5. A method as claimed in claim 3 or claim 4, wherein alteration of the third reagent is amplified by cascaded enzyme amplification or crossed feedback enzyme amplification.

6. A method as claimed in any one of claims 1 to 5, wherein the analyte is a hapten.

7. A method as claimed in any one of claims 1 to 6, wherein the displacement surface is provided by a layer of fibres overlying the detection surface.

8. A method as claimed in claim 7 wherein the layer is a woven fabric.

9. A method as claimed in claim 7 or claim 8, wherein some fibres or particles of the layer carry the immobilised first reagent to which the second reagent is reversibly bound, and other fibres or particles carry a substance which reacts directly or indirectly with the second reagent to generate a reaction product which reacts directly or indirectly with the third reagent on the displacement surface.

10. A method as claimed in claim 9, wherein the second reagent is a conjugate of a first enzyme, and said other fibres or particles carry a second enzyme by means of a linker which is cleavable by the first enzyme.

11. A method as claimed in any one of Claims 1 to 6, wherein the displacement surface is provided by a body of particles.

Fig.1 (a)

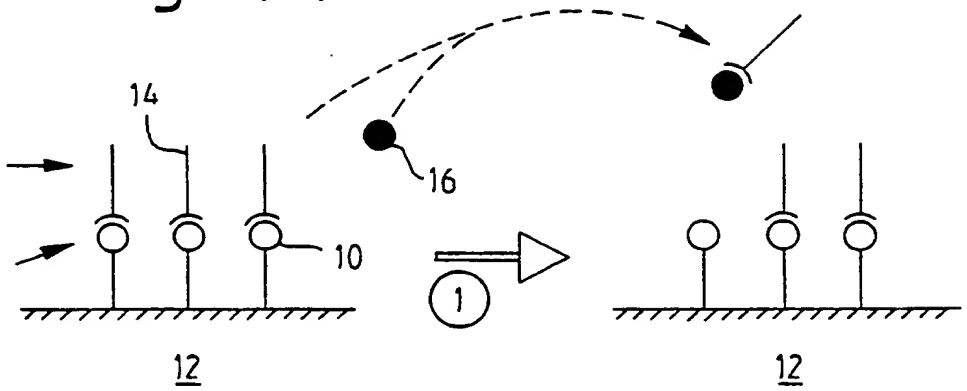


Fig.1(b)

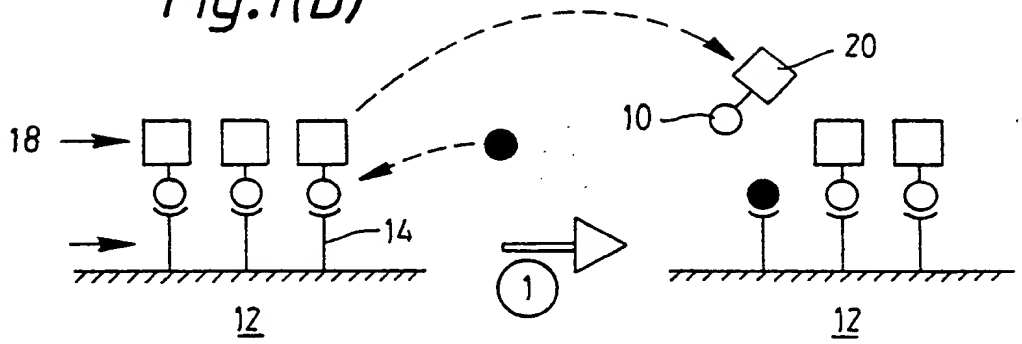


Fig.2(a)

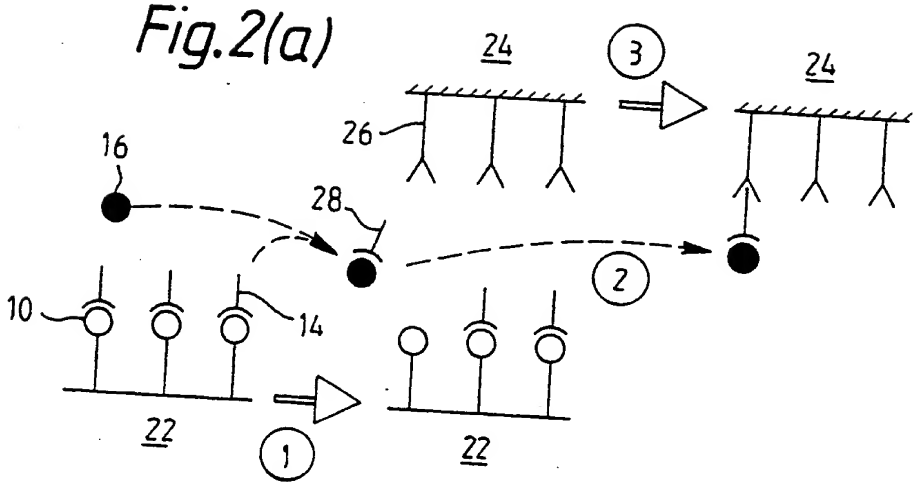


Fig 2(b)

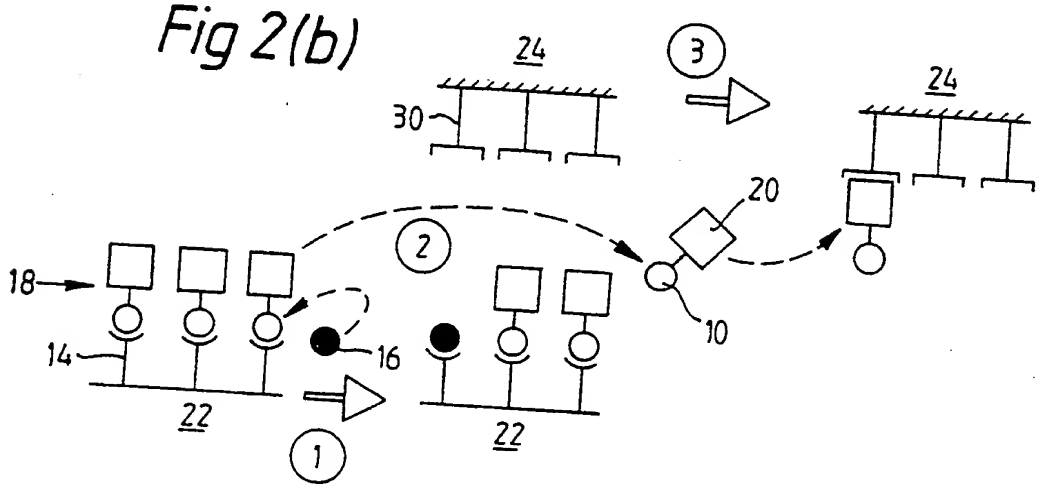


Fig.3

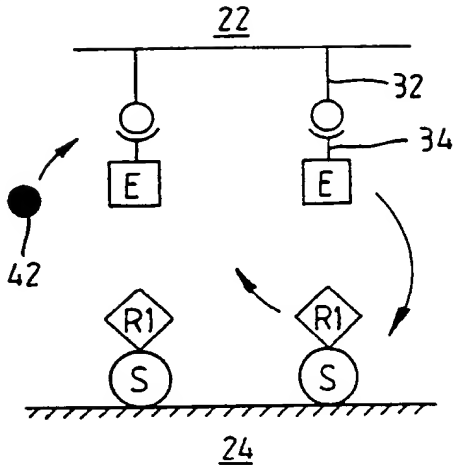


Fig.4

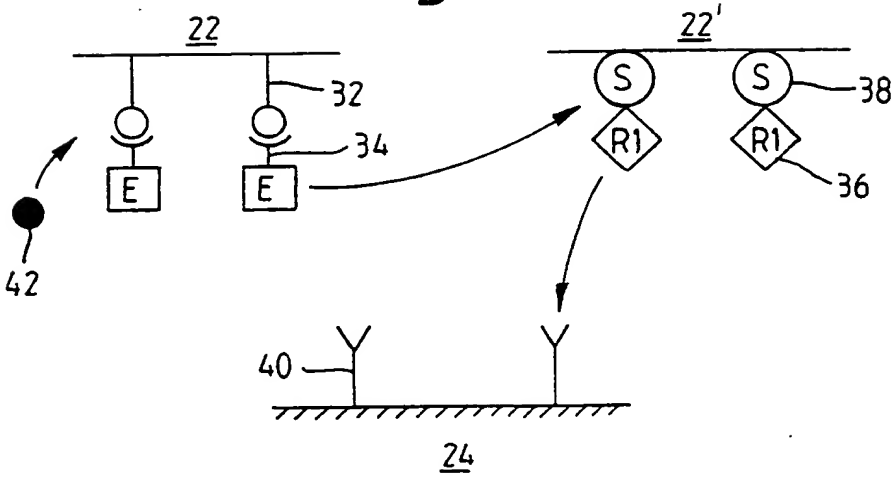


Fig.5

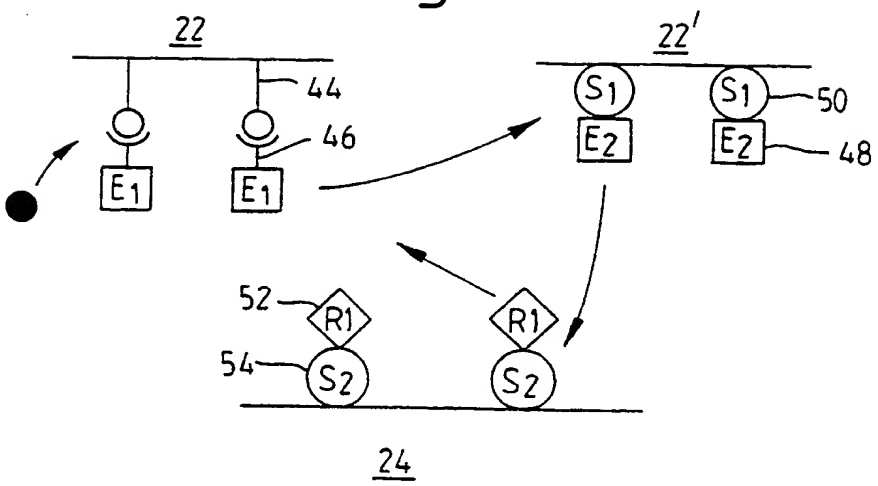
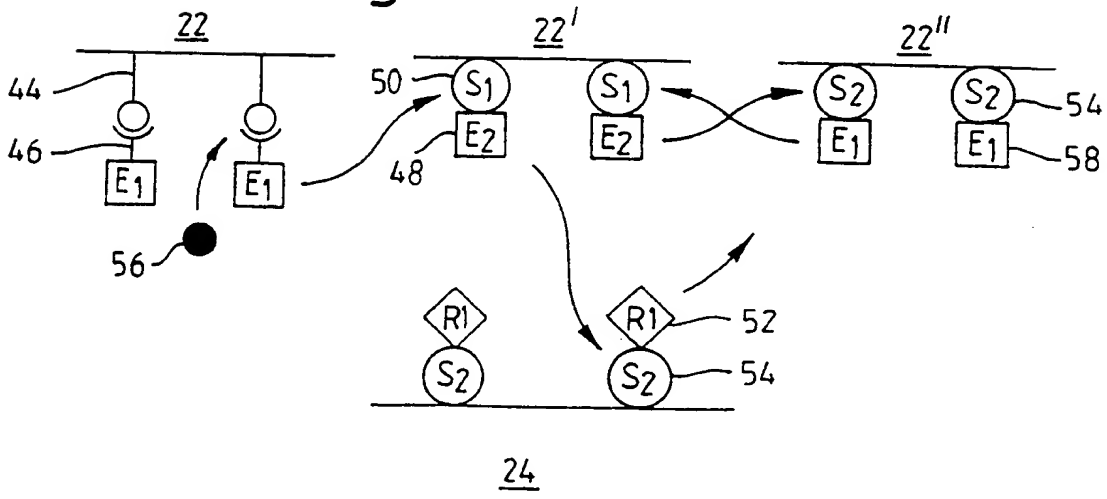


Fig.6



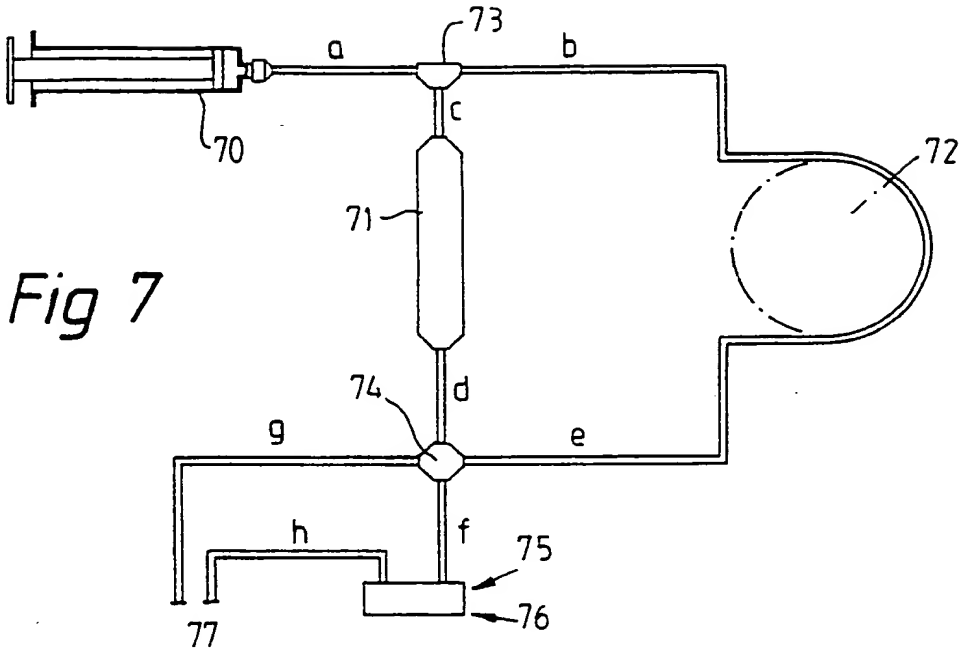


Fig 7

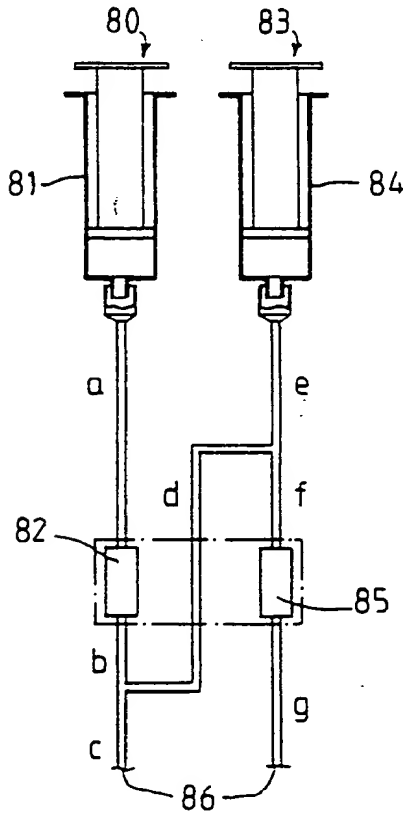


Fig.8